KINETICS OF ACTIVATION OF ADP-RIBOSYLATION AND ADENYLATE CYCLASE BY CHOLERA TOXIN IN CLONED DIFFERENTIATED HEPATOCYTES

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Received 4 September 1978

1. Introduction

Cholera toxin activates adenylate cyclase in a wide variety of eukaryotic cells. This process can be shown to depend upon exogenous NAD^+ in purified rat liver plasma membranes [1], lysed avian erythrocytes [2], mouse neuroblastoma cells [3] and sarcoma 180 membranes [4].

This NAD^+ requirement suggested that ADP-ribosylation might be involved in the activation of adenylate cyclase by cholera toxin, similar to the ADP-ribosylation of elongation factor 2 by diphtheria toxin [5]. Cholera toxin has very recently been shown to catalyze the transfer of the ADP-ribose moiety of NAD^+ to the guanidino moiety of arginine [6], as well as to several membrane components of lysed pigeon erythrocytes, one of which contains the guanyl nucleotide regulatory site of adenylate cyclase [7].

This report examines the kinetics of cholera toxin stimulation of ADP-ribosylation of macromolecules and adenylate cyclase activity in cloned, differentiated, diploid hepatocytes [8] and in a crude membrane preparation derived from these cells, and concludes that there may be a cause-and-effect relationship between both cholera toxin effects.

2. Materials and methods

Purified cholera toxin (Schwarz-Mann) was reconstituted to 1 mg/ml in 50 mM Tris buffer (pH 7.5) containing 1 mM Na_2EDTA, 3 mM NaN_3 and 200 mM NaCl.

RL-PR-C hepatocytes were maintained in monolayer culture as in [8]. A crude plasma membrane preparation was employed for adenylate cyclase and ADP-ribosyltransferase assays. Cells were washed twice with phosphate buffered saline (PBS), scraped from the flask with a rubber policeman into 1 mM $NaHCO_3$, and homogenized in a Teflon–glass homogenizer (1000 rev./min 10 strokes). The homogenate was centrifuged at $4000 \times g$ for 10 min and the pellet washed once with 1 mM $NaHCO_3$. The pellet was resuspended in $NaHCO_3$ to final protein conc. 10–15 mg/ml. The entire procedure was carried out at 4°C. Protein was determined by the Lowry method [9].

Adenylate cyclase was assayed essentially as in [10]. Cyclic AMP was determined in hepatocytes as in [11]. Briefly, cells were scraped from 60 mm petri dishes (about 0.5×10^6 cells) into 0.25% acetic acid. The suspension was heated at 100°C for 3 min, cooled to 4°C, and centrifuged. Aliquots (400 μl) of supernatant fluids were assayed for cAMP by modifications of competitive protein binding assays [12,13] which involve the use of hydroxyapatite to adsorb protein-bound [^{3}H]cAMP.

ADP-ribosyltransferase activity was determined by incubating [*adenine*-2, 8-^{3}H]NAD^+ with hepatocytes or the plasma membrane preparation for the time specified at 37°C. The reaction was ended by the addition of ice-cold 10% trichloroacetic acid (TCA). TCA-insoluble material was collected on Millipore HAWP filters (0.45 μm), washed 3 times with ice-cold 5% TCA, and counted in a commercial liquid scintillation counting mixture (PCS, Amersham).

3. Results and discussion

Cholera toxin binds to a cell surface receptor which appears to contain ganglioside GM_1 [14]. Binding is probably multi-valent, and involves the B subunits of the toxin [15]. In RL-PR-C hepatocytes, cAMP accumulation in response to cholera toxin proceeded following a 45–60 min lag (fig.1). After this lag, presumably necessary for the binding of the toxin and penetration of the plasma membrane by the A subunits [16,17], cAMP continued to accumulate for at least 150 min. At this point, cAMP levels in toxin-stimulated cultures reached 10-times basal values.

Since we observed that the activation of adenylate cyclase by cholera toxin in a washed hepatocyte plasma membrane preparation required exogenous NAD^+ (unpublished observations), we studied the effect of cholera toxin on the incorporation of labeled NAD^+ into TCA-insoluble macromolecules in both intact hepatocytes and the plasma membrane preparation. Cholera toxin stimulated the incorporation of [*adenine*-2,8-^{3}H]NAD^+, but not [*nicotinamide carbonyl*-^{14}C]NAD^+, into cell macromolecules (table 1); the label distribution indicates that it was the ADP-R moiety of NAD^+ that was incorporated.

The kinetics of formation of the ADP-ribosylated product by cholera toxin was determined in intact hepatocytes. Although considerable basal ADP-ribosylation into as yet unknown products occurred in these cells, a substantial increment in ADP-ribosylation due to cholera toxin was observed. Cholera toxin-catalyzed ADP-ribosylation was evident by 15 min and continued even after basal ADP-ribosylation had leveled off (fig.2). It is clear, therefore, that in intact hepatocytes, toxin-catalyzed ADP-ribosylation preceded toxin-induced cAMP accumulation.

Table 1
Specificity of incorporation of NAD^+ moieties into hepatocyte macromolecules

Substrate	NAD^+ incorporated (pmol/10^6 cells)	
	Basal	+ Cholera toxin
[*adenine*-2,8-^{3}H]NAD^+	11.3	16.9
[*nicotinamide carbonyl*-^{14}C]NAD^+	0.2	0.8

Cells in monolayer were incubated in 1.0 ml PBS containing 1.0 mM dithiothreitol and 170 nM labeled NAD^+, ± 5 μg/ml cholera toxin, for 1 h at 37°C, after which TCA-insoluble counts were quantified as in section 2. Each value represents the average of triplicate incubations

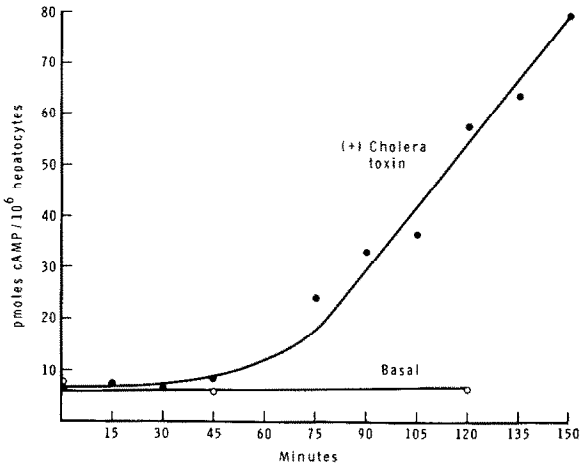

Fig.1. Kinetics of cholera toxin-stimulated cAMP accumulation in RL-PR-C hepatocytes. Cells in monolayer were incubated with cholera toxin (1.68 μg/ml) in PBS for the specified times. Each point is the average of cAMP assays of triplicate incubations.

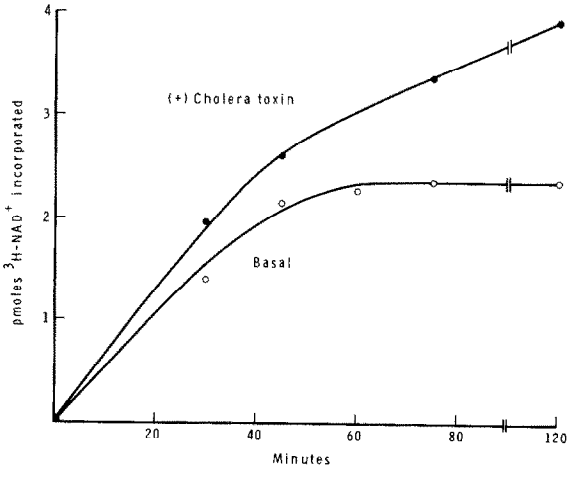

Fig.2. Kinetics of ADP-ribosylation of hepatocytes. Cells in monolayer were incubated with 1.68 μg/ml cholera toxin, 1.0 mM dithiothreitol, and 338 nM [*adenine*-2,8-^{3}H]NAD^+ in PBS for the specified times, and TCA-insoluble material isolated. Each point represents the results from duplicate incubations.

Control experiments conducted in the absence of tissue established that the toxin did not catalyze its own ADP-ribosylation, in contrast to [18].

The hepatocyte macromolecules covalently labeled from [^{3}H]NAD$^+$ by cholera toxin exhibited characteristics of a protein linked to ADP-ribose via the pentose moiety. Although the TCA-insoluble radioactive materials were stable to brief heating in dilute acid, similar treatment with 1 N NaOH solublized over 75% of the counts. In addition, incubation with trypsin for 30 min at 37°C reduced TCA-insoluble counts by 66%. Lability to alkali is characteristic of the linkage between ribose and the guanidino group of arginine, and arginine has been shown to be an acceptor in the ADP-ribosyltransferase reaction catalyzed by cholera toxin [6].

In hepatocyte plasma membranes, cholera toxin activated adenylate cyclase without the lag period observed in intact cells (fig.3), reflecting perhaps, the greater accessibility of adenylate cyclase to the toxin in disrupted plasma membranes [1,16]. Despite the absence of a prolonged lag period, the specific adenylate cyclase activity was lowest at 5 min, highest between 10–15 min and reduced thereafter (fig.3). In contrast, cholera toxin's specific ADP-ribosyltransferase activity, assayed under the same conditions employed for adenylate cyclase assays, reached a maximum at 5 min and fell off rapidly thereafter.

These studies support the theory that cholera toxin catalyzes the ADP-ribosylation of one or more components of plasma membranes as an early part of the mechanism by which it activates adenylate cyclase. The hepatocyte membrane components ADP-ribosylated by the toxin in the present studies may be similar to the 3 membrane components of pigeon erythrocytes whose ADP-ribosylation from NAD$^+$ by cholera toxin has recently been reported; one of the 3 membrane components appears to be identical to the GTP regulatory components of the adenylate cyclase system of avian erythrocytes [7]. We are attempting to identify the acceptors for cholera toxin-catalyzed ADP-ribosylation in differentiated, dividing hepatocytes.

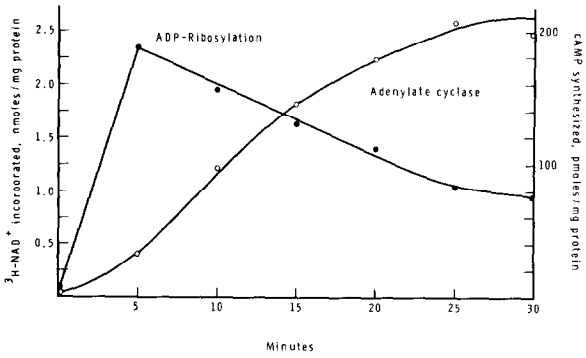

Fig.3. Kinetics of activation of ADP-ribosylation and adenylate cyclase by cholera toxin in hepatocyte membranes. Adenylate cyclase activity of membranes (1.5 mg protein/ml) was assayed with standard incubation mixture components and conditions [10], but with added 1.0 mM NAD$^+$ and 1.68 μg/ml cholera toxin. ADP-ribosylation was assayed under the identical incubation conditions, except that the ATP was unlabeled and NAD$^+$ was replaced by [*adenine*-2,8-^{3}H]NAD$^+$ (3.3 μM).

Acknowledgements

This work was supported by NIH Grant AM-05475 END. to M.B. and is taken from a PhD thesis to be submitted by S.B. to Georgetown University.

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